SPECIFIC FLUORESCENT LABELING OF CHICKEN MYOFIBRIL Z-LINE PROTEINS CATALYZED BY GUINEA PIG LIVER TRANSGLUTAMINASE

DAVID L. GARD and ELIAS LAZARIDES

From the Division of Biology, California Institute of Technology, Pasadena, California 91125

ABSTRACT

Guinea pig liver transglutaminase has been found to catalyze the covalent incorporation of dansylcadaverine into chicken skeletal muscle myofibril proteins. Epifluorescence microscopy reveals that the incorporated dansylcadaverine is specifically localized at or near the myofibril Z line. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) indicates that actin constitutes a major fraction of the labeled material; the Z-line proteins α-actinin and desmin also show significant labeling, as well as tropomyosin, several additional unidentified proteins, and material with an extremely high molecular weight. The Z-line-specific fluorescence can be removed by brief trypsinization, which releases fluorescent α-actinin into the supernate. The majority of the fluorescent protein species are resistant to extraction by either 0.6 M KCl or KI. These results, in conjunction with the microscopic localization, suggest that the dansyl-labeled proteins are constituents of the myofibril Z line.

A significant amount of fluorescently labeled transglutaminase is also present in labeled myofibrils, which is resistant to extraction with either 0.6 M KCl or KI. This result indicates a strong, noncovalent interaction between the transglutaminase molecule and the myofibril Z line.

KEY WORDS actin · desmin · α-actinin

The myofibrillar Z line serves as the anchor point for the lattice of actin filaments in a sarcomere. As such, it is responsible for transmitting the forces generated within one sarcomere to adjacent sarcomeres, as well as providing a structural framework upon which the actin thin-filament lattice can be organized. These roles have led to an extensive investigation of the morphology and ultrastructure of the Z line and its molecular components.

The molecular constituents of the Z line have been under intense investigation, because an understanding of their properties may provide insight into the molecular organization of this structure. The sensitivity of the vertebrate muscle Z line to proteolytic digestion suggests that the major constituents of the Z line are proteinaceous (17, 23, 44). However, few proteins have been unambiguously identified as components of this structure. α-Actinin is, perhaps, the most conclusively demonstrated Z-line protein, localized by immunological techniques (24, 26, 32), and extraction procedures. Desmin, the 50,000-dalton subunit of the intermediate (100 Å) filaments in smooth muscle, has also been shown to be localized at the Z line of skeletal muscle myofibrils by immunoflu-

J. CELL BIOLOGY © The Rockefeller University Press · 0021-9525/79/05/0336/12 $1.00
Volume 81 May 1979 336-347

orescence (28). Tropomyosin has been suggested as a Z-line component, but no conclusive evidence for its localization there has been presented to date (11, 21, 32, 44). Several other proteins of unknown identity have been implicated in Z-line structure of vertebrate and invertebrate myofibrils by using techniques of differential extraction (5, 11, 12, 38). Unfortunately, the differences in myofibril source and the uncertainty of such extraction procedures makes interpretation of these results somewhat difficult.

The transglutaminases (TGases) are a class of enzymes which catalyze a Ca^{++}-dependent acyl transfer reaction between primary amine donors and peptide-bound glutamine acceptors (39; for review, see [14]). These enzymes have been implicated in several physiological roles: a TGase found in hair follicles has been postulated to cross link hair keratin (7); clotting factor XIII is a multisubunited plasma TGase essential for the cross linking of fibrin in clot formation (29); and a monomeric form of factor XIII is found in platelets, and may serve similar functions as plasma factor XIII (41). The dipeptide ϵ-(γ-glutamyl)lysine has been identified in the cornified envelope of epidermal keratinocytes in culture (35, 36) and in the stratum corneum (1), thus implicating a TGase activity in the terminal differentiation of epidermal tissues. The ϵ-(γ-glutamyl)lysine dipeptide has also been identified in clotted guinea pig semen (46) and in membrane protein fractions from cultured mouse cells (3), thus suggesting the action of a TGase at these sites. A Ca^{++}-activated aggregation and cross linking of erythrocyte membrane proteins has been reported, and is similarly attributed to a TGase (2, 31). The widespread occurrence of TGase activities or their cross-linked protein products in many tissues from various sources suggests that the cross linking of protein may be a ubiquitous phenomenon, with an important role in determining the mechanical integrity of many cellular and extracellular structures.

An acyl transfer activity has been reported in smooth, cardiac, and skeletal muscle, which appears to be antigenically similar to the TGase isolated from liver (6). In the course of an investigation into the possible involvement of a TGase activity in muscle, we have studied the effect of exogenously added guinea pig liver TGase as a means of covalently attaching small fluorescent molecules to fibrillar proteins (13, 22, 30).

In this paper, we report that guinea pig liver TGase incorporates the lysine analogue dansylcadaverine (DCAD) into proteins of isolated chicken skeletal muscle myofibrils. Furthermore, under the appropriate reducing conditions, the incorporation of fluorescent label appears to be specific to proteins located at or near the myofibril Z line, as indicated by epifluorescence microscopy. This observation allowed us to use this technique as a probe to analyze the components of the Z line.

MATERIALS AND METHODS

Materials

Eastman Organic Chemicals Div., Eastman Kodak Co. (Rochester, N. Y.) supplied acrylamide for slab gels (No. 5521) and isoelectric focusing gels (No. X5521), and *N,N'*-methylenebis acrylamide (No. 8383). Bovine serum albumin, crystallized, was obtained from Miles Laboratories Inc., Miles Research Products (Elkhart, Ind.). Sigma Chemical Co. (St. Louis, Mo.) supplied glutathione, monodansylcadaverine, Tris, soybean trypsin inhibitor, and assorted protein gel standards. Nonidet P-40 is a trademark of the Shell Chemical Co. (Houston, Tex.). Sodium dodecyl sulfate (SDS) was from Serva (Heidelberg, Germany). Ampholines were from LKB Produkter (Bromma, Sweden). Ultrapure urea was obtained from Schwarz/Mann Div., Becton, Dickinson & Co., (Orangeburg, N. Y.). Worthington Biochemical Corp. (Freehold, N. J.) supplied trypsin TPCK. New England Nuclear (Boston, Mass.) supplied [^{14}C]-cadaverine. Unless otherwise mentioned, all other chemicals were reagent grade.

The phosphate-buffered saline (PBS) used throughout these experiments contains 0.137 M NaCl, 2.7 mM KCl, 9.6 mM Na-K phosphate, adjusted to pH 7.4.

Methods

MYOFIBRIL PREPARATION: White leghorn chicken leg muscles were cleaned of fat and loose connective tissue, stretched longitudinally, and secured to wooden applicators with surgical suture. These preparations were then placed in 50% glycerol containing 1/10 × PBS (pH 7.4) (described above), and 1 mM EGTA, and were stored at −10°C for at least 2 wk before use. Preparations of individual myofibrils and myofibril bundles were obtained by blending glycerinated muscle for 3–5 min in glycerol 1/10 × PBS buffer, by using the top speed of a Lourdes MM-1B homogenizer (Lourdes Div., Vernitron Medical Products, Inc., Carlstadt, N. J.). The resulting myofibril suspensions were filtered through 1-ply cheesecloth to remove large chunks of muscle, and stored at −10°C until use. Just before use, myofibrils were washed three times in a total of 50 vol of 1 × PBS by repeated suspension-centrifugation. The final myofibril pellet was resuspended in 1 vol of PBS.

TGASE ISOLATION: Guinea pig liver TGase was

isolated by the method of Connellan et al. (8). Enzyme activity was monitored by measuring the incorporation of [^{14}C]cadaverine into α-casein, or by the hydroxamate assay described by Folk (15). Protein concentrations were measured by a modification of the Lowry method (41). TGase purity was estimated to be ~90–94% by mass, based upon densitometry of Coomassie brilliant blue (CBB)-stained polyacrylamide gels (see below); several low molecular weight contaminants were observed, but were judged to be inconsequential to the results reported below. TGase was stored at a concentration of ~0.3 mg/ml in a buffer containing 0.16 M KCl, 10 mM Tris acetate, pH 6.0, 1 mM EDTA, and 1 mM sodium azide (KCl buffer) at 4°C for several months without significant loss of activity.

MYOFIBRIL LABELING: 30 μg TGase in 100 μl of KCl buffer (see TGase Isolation) was added to 100 μl of myofibril suspension, along with 100 μl of a labeling buffer containing 7.5 mM DCAD, 50 mM Tris-HCl, pH 7.5, and 6 mM glutathione (GSH), giving a final DCAD concentration of 2.5 mM, and 2 mM final GSH concentration. $CaCl_2$ was added to 5 mM to initiate the TGase-catalyzed labeling reaction; controls received EDTA, in place of $CaCl_2$, to a final concentration of 10 mM. Samples were then incubated 1.5–2 h at 37°C. The labeling reaction was terminated by the addition of 1.0 ml PBS containing 10 mM EDTA, and the myofibrils were washed 3 × with 1 ml vol of PBS plus EDTA. The final myofibril pellets were suspended in 100 μl PBS (no EDTA), and were processed for microscopy and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The effect of glutathione concentration upon labeling specificity was ascertained by substituting 3, 6, 15, 30, or 60 mM GSH in the reaction medium, yielding final concentrations of 1, 2, 5, 10, and 20 mM during the labeling reaction.

PHASE/FLUORESCENCE MICROSCOPY OF LABELED MYOFIBRILS: Myofibrils were placed on glass slides, and coverslips were affixed with Elvanol (E. I. DuPont de Nemours & Co., Wilmington, Del.). Samples were observed using a Leitz Orthoplan microscope (E. Leitz, Inc., Rockleigh, N. J.) equipped with N100×, 1.32 Numerical Aperture (N. A.) phase-fluorescence lens and filter module A (wide spectrum UV) for dansyl fluorescence. Results were recorded on Kodak Tri-X pan film exposed at ASA 800, and developed in Diafine (Accufine, Inc., Chicago, Ill.). Magnification was calibrated by photographing the rulings of a hemocytometer.

ONE-DIMENSIONAL SDS-PAGE: One-dimensional electrophoretic analysis was performed on a high resolution SDS-polyacrylamide slab gel system as previously described (20). The stacking gel contained 5% acrylamide, 0.13% *N,N'*-methylene bisacrylamide (Bis), 0.125 M Tris-HCl, pH 6.8, and 0.1% SDS. Both 12.5% acrylamide and 17.5% acrylamide resolving gels were employed, with 17.5% gels giving resolution down to 16,000 daltons. 17.5% acrylamide gels contained 0.074% Bis, 0.387 M Tris-HCl, pH 8.6, and 0.1% SDS. Polymerization was catalyzed by the addition of 100 μl of 10% ammonium persulfate, and 10 μl of *N,N,N',N'*-tetramethylethylene diamine/30 ml of gel solution. Samples were diluted 1:1 in 2 × SDS sample buffer containing 0.08 M Tris-HCl, pH 6.7, 10% glycerol, 2% SDS, 0.1 M dithiothreitol, and bromphenol blue.

Gels were observed for fluorescence over a long-wave UV light source, and photographed on Polaroid PN-55 film (Polaroid Corp., Cambridge, Mass.), using an orange filter to block background illumination.

Gels were then stained overnight in 50% ethanol, 10% acetic acid containing 0.1% CBB R-250, and were destained for 2 d in three changes of 10% ethanol, 5% acetic acid. Stained gels were photographed over a light box by using Polaroid PN-55 film with an orange filter to enhance contrast.

TRYPSIN DIGESTION: 100 μl of a suspension of (50 μl packed) labeled myofibrils were incubated 5, 10, or 30 min with 5 μg trypsin. Digestion was terminated by the addition of 20 μg soybean trypsin inhibitors (STI) in 200 μl PBS. The digested myofibrils were pelleted at 1,000 *g*, and the supernate was prepared immediately for SDS-PAGE. After two additional washes with STI in PBS, samples of the digested myofibrils were prepared for microscopy and SDS-PAGE.

HIGH SALT (KCL OR KI) EXTRACTIONS: High salt extractions of labeled myofibrils were accomplished with either 0.6 M KCl containing 20 mM sodium pyrophosphate, 10 mM Tris-HCl, pH 7.5, and 5 mM EDTA, or 0.6 M KI containing 20 mM sodium thiosulfate, 10 mM Tris-HCl, pH 7.2, and 5 mM EDTA. Prelabeled myofibrils were pelleted by centrifugation, then suspended in 2 vol of one of the above extraction buffers, and incubated at room temperature; though extraction proceeds very rapidly, extraction times were set at 45 min. At the end of the exraction period, samples were pelleted and the supernate was removed and processed for SDS-PAGE. The high salt residues were washed briefly with the appropriate extraction buffer, and were processed for microscopy and SDS-PAGE.

RESULTS

DCAD Labeling of Myofibrils

Fig. 1 shows the characteristic labeling pattern seen when myofibrils are incubated with DCAD and TGase in the presence (Fig. 1 *c–d*) or absence (Fig. 1 *a–b*) of Ca^{++} ions. Note the extreme specificity of the reaction, with preferential labeling of the Z line, to the exclusion of the remainder of the myofibril. The control myofibrils (minus Ca^{++}, Fig. 1 *b*) show a total lack of DCAD incorporation. Similarly, in the absence of exogenous TGase, no DCAD incorporation is observed

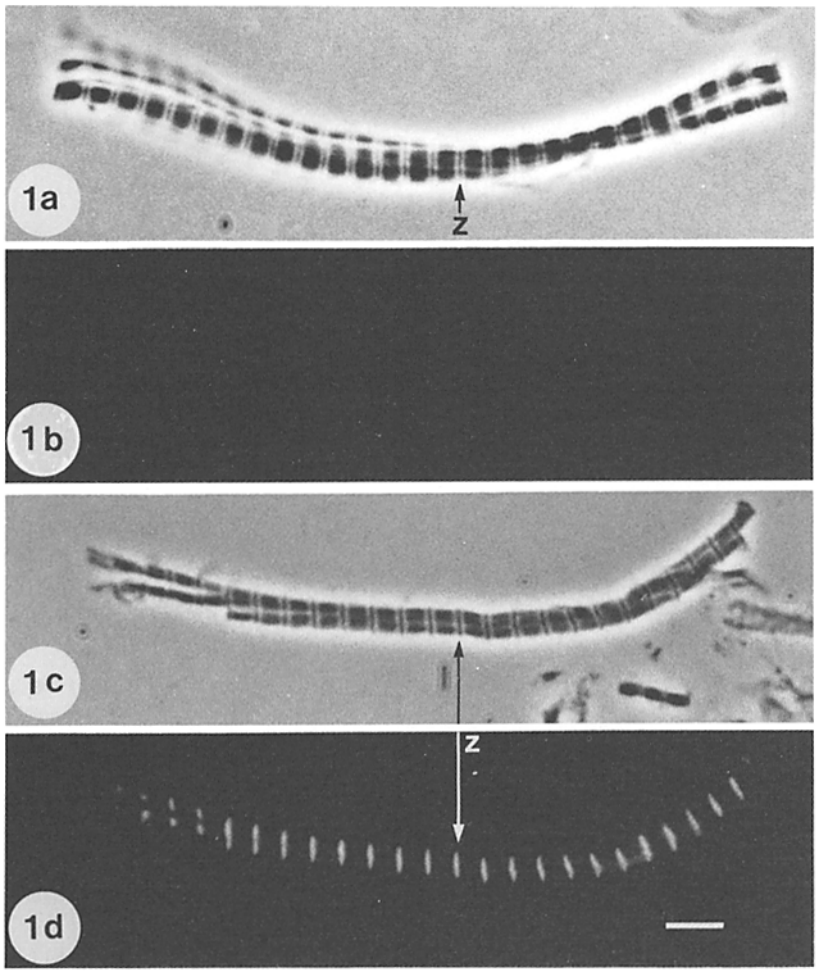


FIGURE 1 Comparison of myofibrils incubated with transglutaminase and DCAD in the presence or absence of Ca^{++} ions (see Methods). (*a*) Phase microscopy of control ($-Ca^{2+}$) myofibrils, showing prominent Z lines (arrow), and A and I zones. (*b*) Fluorescent microscopy of the same myofibril bundle, showing the lack of any fluorescent labeling in the absence of Ca^{2+}. (*c*) Phase microscopy of a labeled ($+Ca^{2+}$) myofibril bundle shows normal myofibril structures. (*d*) Fluorescent microscopy of the identical labeled bundle shows characteristic Z-line fluorescence (arrow). Bar, 5 μm. × 1,600.

(data not shown). Occasionally weak M-line or central A-zone labeling is detectable, though such labeling is observed in only a minor fraction of myofibrils. The percentage of myofibrils showing such a component appears to be related to the concentration of reducing agent in the reaction medium.

When myofibrils are DCAD labeled under conditions which minimize or eliminate M-line labeling (2 mM GSH), washed extensively with PBS, and subjected to SDS-PAGE, the fluorescent proteins which account for the Z-line label may be visualized and analyzed. Fig. 2 shows the results of such a gel, photographed with UV illumination, and again after CBB staining. Approx. 14 fluorescent bands can be resolved in the mol wt range of 15–200,000, of which 11 are invariably present in all preparations. Additional washing of myofibrils, up to 8 times with PBS, and extraction with 0.5% NP-40 for 15 min before labeling has no effect on the pattern of labeled proteins (data not shown). Purification of the isolated myofibrils by preparative centrifugation on a 40–70% sucrose gradient depleted only one labeled band, a doublet at ~220,000, which has been tentatively identified as cross-linked dimeric collagen because of its

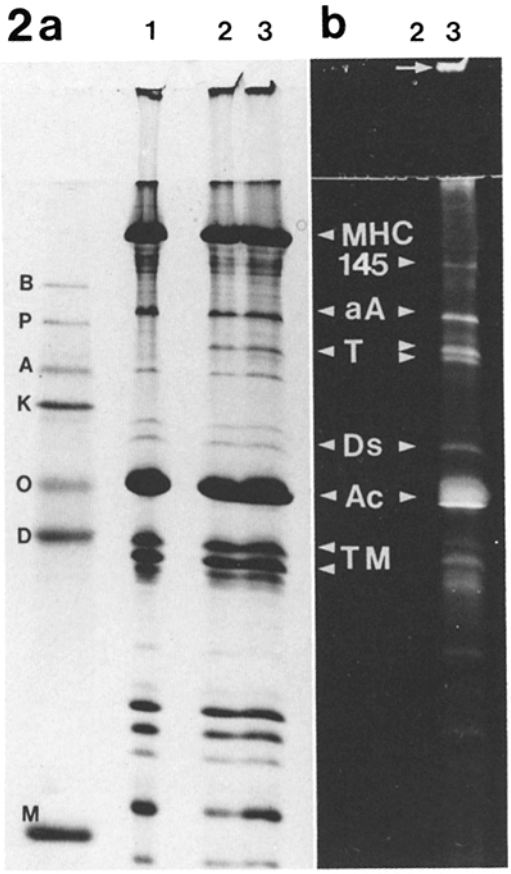


FIGURE 2 SDS-PAGE analysis of DCAD-labeled myofibril proteins. Myofibrils were labeled as described (see Methods), and electrophoresed on a 17.5% acrylamide gel. (*a*) Shows the CBB-stained gel, with a series of protein standards to the extreme left. *B*: β-galactosidase (130,000); *P*: Phosphorylase A (94,000); *A*: Bovine serum albumin (68,000); *K*: Pyruvate kinase (57,000); *O*: Ovalbumin (43,000); *D*: D-amino acid oxidase (37,000); *M*: Myoglobin (17,200). (*b*) Shows the fluorescent photograph of the same gel, before staining. Gel lanes in (*a*) and (*b*), and in all subsequent stained fluorescent gel pairs, are numbered identically. Lane *a1* shows the protein components of untreated myofibrils for comparison. Lanes *2* (*a2* and *b2*) show myofibrils incubated with TGase and DCAD in the absence of Ca^{2+}. Note the total lack of DCAD incorporation into the myofibril proteins. Lane *3* (*a3* and *b3*) show the incorporation of label in the presence of Ca^{2+}. The fluorescent protein bands corresponding to α-actinin (*aA*, 102,000), TGase (*T*, doublet of 82,000), desmin (*Ds*, 50,000), actin (*Ac*, 42,000), and tropomyosins (*TM*, ~35,000) are quite distinct. In addition, there is a band corresponding to a mol wt of 145,000, and some minor bands corresponding to mol wt of 87,000 (just above TGase) and mol wt <20,000. A large amount of fluorescent material is seen at the top of the stacking gel (arrow) which may represent cross-linked protein. Note the lack of myosin (*MHC*) fluorescence.

violet staining by CBB (data not shown). The major bands, based upon fluorescent intensity, possess apparent mol wt of 145,000, 102,000 (α-actinin), 81,000 (TGase), 50,000 (desmin), and 42,000 (actin). Four less intensely labeled bands correspond to masses of 35,000, 32,000, 25,000, and 21,000 daltons. The 35,000- and 32,000-dalton components appear to be tropomyosins. Components of 87,000 and 61,000 daltons are labeled in some preparations, but have not been conclusively identified, and may represent proteolytic cleavage fragments of other Z-line proteins.

The fluorescent doublet near 81,000 has been identified as transglutaminase, based upon comigration with purified TGase. It has been reported previously that TGase will incorporate DCAD into itself (4). The production of a doublet may be caused by variations in the labeling ratio of the enzyme molecules, or by modifications of different glutamine residues; such heterogeneous modifications may result in the observed disparity of the enzyme's mobility. The presence of TGase in SDS-PAGE analysis of labeled myofibrils suggests that TGase may bind to isolated myofibrils. Because the TGase is labeled with DCAD, and because only the myofibril Z lines show label incorporation, it is assumed that the labeled TGase is bound at the myofibril Z lines.

Fluorescent bands of high intensity are seen at the top of the 4% stacking gel and the resolving gel, indicating that this material has a very high molecular weight. Because TGase catalyzes the incorporation of label into protein bound glutamine residues only (14), we assume that this material is proteinaceous. TGase is known to covalently cross link proteins, including itself, to form high molecular weight oligomers (4, 9); however we have not detected the formation of any TGase oligomers, or oligomers of known myofibril proteins, during our labeling procedure. Because high molecular weight material is also seen in SDS-PAGE of unlabeled myofibrils, we believe that this TGase-labeled high molecular weight protein material exists before the labeling reaction and may represent proteins covalently cross linked by an endogenous muscle TGase.

The identity of labeled myofibril proteins was further established by using two-dimensional isoelectric focusing SDS-PAGE as described by O'Farrell (33) and modified by Hubbard and Lazarides (20). The major labeled protein is readily identified as actin, though some heterogeneity is observed in the first (isoelectric focusing [IEF]) dimension (data not shown). The 50,000-dalton species can be conclusively identified as desmin by its molecular weight and isoelectric point. Desmin

consists of two isoelectric variants, α and β, which differ by ~0.05 pH units and are slightly more basic than actin (25). Both desmin variants are seen to be fluorescently labeled, with approximately equal intensity. Labeled α-actinin and TGase do not focus well in IEF gels at loadings sufficient to visualize their fluorescence, and give rise to streaks identifiable by their respective molecular weights.

The 145,000-dalton protein seen in one-dimensional SDS-PAGE is visible as a cluster of 4–6 well resolved spots slightly more basic than α- and β-desmin. The precise identities of these proteins remain unknown and are under investigation (see Discussion).

The DCAD-labeled protein species with molecular weights less than actin, which are readily apparent in one-dimensional SDS-PAGE, are only weakly detectable in IEF/SDS-PAGE. The reason for this is unclear, though it may be because of a lowered solubility in the presence of urea. Very faint fluorescence is associated with at least one tropomyosin variant.

Trypsin Digestion of Labeled Myofibrils

Brief digestion with trypsin has been shown to remove the Z-line phase density from isolated myofibrils (17, 23, 43). We have used trypsinization of DCAD-labeled myofibrils to further characterize the Z-line specificity of the labeling reaction. Epifluorescence microscopy showed a progressive loss of Z-line fluorescence concomitant with a loss of Z-line phase density. There was great variability in the extent of digestion of individual myofibrils, however. As the time of digestion increased, myofibrils tended to separate at the level of the Z line, breaking into units of one or more sarcomeres in length. Such myofibrils were devoid of Z-line phase density and had little or no detectable Z-line fluorescence (see Fig. 4). Even with extensive digestion, some fibrils retained a very faint fluorescence while lacking a detectable Z-line phase density. The detection of low levels of fluorescence in such myofibrils might indicate the presence of residual undigested Z-line material which is not resolved by phase microscopy.

SDS-PAGE analysis of digested labeled myofibrils and of the digestion supernate (Fig. 3) shows that several fluorescent peptides are released into the supernate during trypsinization, of which one with mol wt 102,000 is the most prominent. The latter peptide comigrates with undigested α-actinin and appears to be cleaved subsequent to its release, because it is much less prevalent in supernates from more extensive digests. CBB staining indicates that considerable protein is released into the supernate, including actin, myosin, and tropomyosin. Some of these A- and I-band components may actually be present as short myofibril fragments which are not pelleted by the low speed centrifugation used.

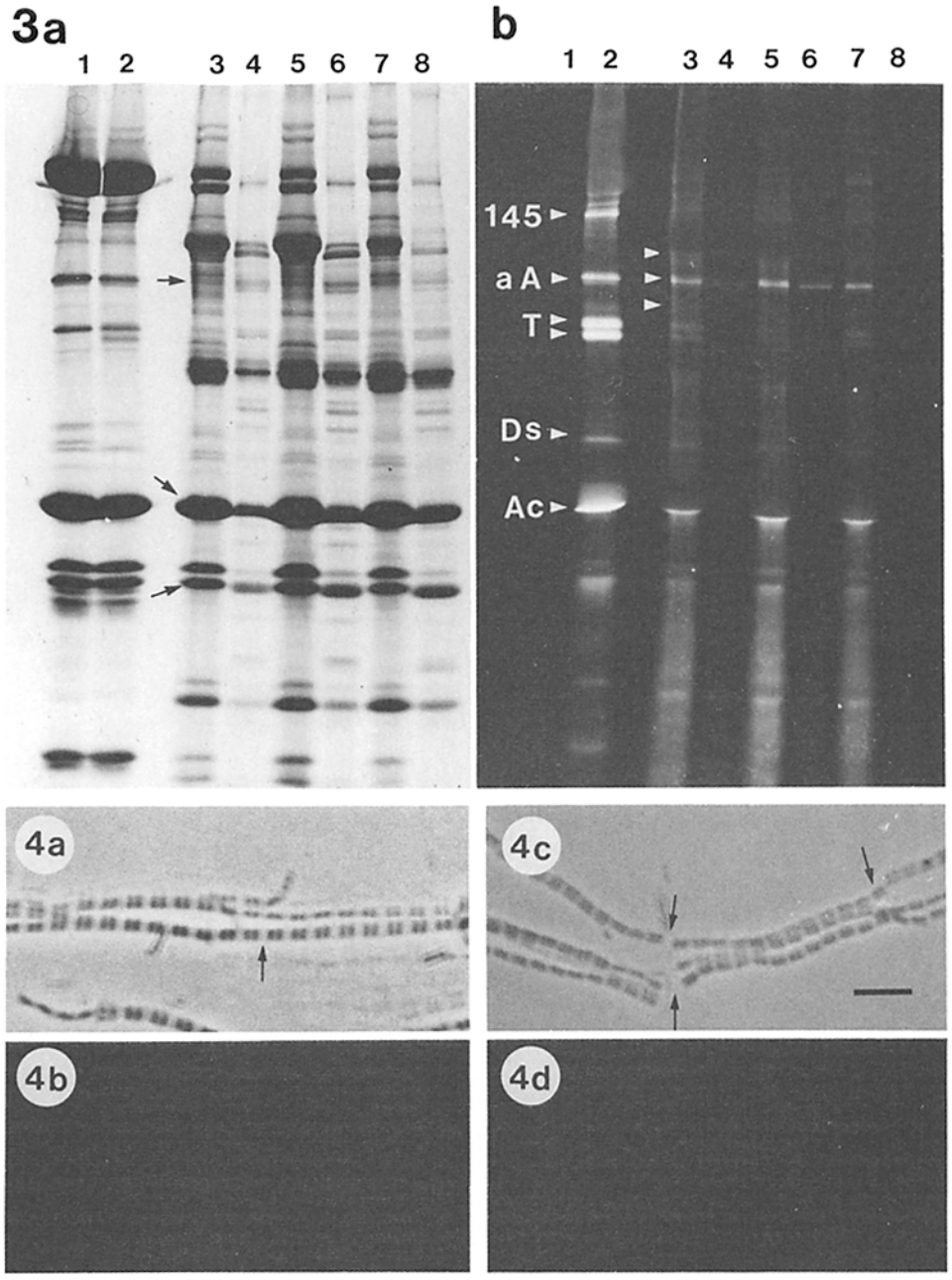
Analysis of the tryptic residues shows a depletion of several fluorescent bands, including desmin, actin, and tropomyosin, with the appearance of a fluorescent smear at the lower end of the gel. The lack of total depletion probably relates to the variation in the extent of digestion of individual myofibrils, as discussed above.

High Ionic Strength Extractions

High ionic strength buffers have been shown to extract a large percentage of myofibril actomyosin, and the loss of A- and I-band structures are visible by phase microscopy (21). The remaining residue consists of the myofibril Z disks, with some actomyosin condensed upon them, held in linear arrays by an as yet unidentified component (19). When the labeled myofibrils are extracted with 0.6 M KI, the Z-line residues show characteristic fluorescence which is not visibly weaker than in unextracted myofibrils (see Fig. 5*b*).

SDS-PAGE analysis of the KI supernates and residues of labeled myofibrils indicates that the majority of fluorescent proteins remain insoluble after a 45-min extraction (Fig. 6). Only small amounts of labeled α-actinin, TGase, actin, tropomyosin, and 145,000-dalton proteins are extracted. No fluorescently labeled desmin is detectable in the KI supernate even at sample loadings much higher than those shown. CBB staining shows that significant amounts of nonfluorescent actin and tropomyosin are extracted, leaving the fluorescent counterparts to these proteins insoluble. Myosin and several other nonfluorescent proteins are also extracted by KI.

Extraction with 0.6 M KCl in the presence of 2 mM pyrophosphate is much less efficient, removing less of the observable myofibril structure while causing some swelling of the A band (see Fig. 5*c*). As in the case of KI extraction, KCl extracted myofibrils retain their characteristic Z-line labeling with no apparent decrease in intensity. SDS-PAGE analysis of KCl supernates and residues (Fig. 6) also shows the inefficiency of the extraction procedure. The only fluorescent protein ex-



tracted in great quantity is the 145,000-dalton protein, with very minor amounts of TGase, actin, and low molecular weight proteins. CBB staining shows the release of small amounts of myosin, the 145,000 protein, actin, tropomyosin, and low molecular weight components.

It is of interest to note that a considerable amount of labeled TGase is detectable in SDS-PAGE analysis of both KCl and KI residues, thus suggesting a strong noncovalent interaction between TGase molecules and myofibril Z lines.

DISCUSSION

Our results show a startling and highly specific labeling of the Z lines of isolated myofibrils. Analysis of labeled myofibrils by SDS-PAGE allows the identification of several proteins to which DCAD has been coupled. Two of the protein species labeled by TGase have been previously shown to be located at the myofibril Z line. α-Actinin, first isolated and studied by Ebashi and Ebashi (10), has been immunologically localized at the Z line with both indirect immunofluorescence (26, 32) and immunoelectron microscope techniques (24). In addition, α-actinin is released from myofibrils by procedures which remove the Z-line phase density, such as low salt extraction (44), deoxycholate (DOC) extraction (11, 12), or trypsin digestion (17). Stromer and Goll have shown that purified α-actinin can reconstitute the Z-line phase density of myofibrils extracted with low salt buffers (42, 44). Together these observations provide substantial evidence that α-actinin constitutes one structural protein of the Z line. It has been suggested that α-actinin may be a component of the amorphous Z-line matrix noted in ultrastructural studies, an idea supported by its actin-binding capacities and solubility properties.

Our results with DCAD-labeled myofibrils are consistent with the above observations. A protein with a mol wt of 102,000 which is highly labeled with DCAD has been identified as α-actinin. This fluorescent protein is released from myofibrils by brief treatment with trypsin, apparently intact, corresponding to previous reports that α-actinin is relatively insensitive to cleavage by trypsin and that an α-actininlike protein is released from myofibrils by trypsinization (7). This labeled α-actinin is also resistant to extraction with 0.6 M KI, which solubilizes much of the myofibrillar actomyosin.

Desmin, the 100-Å filament subunit isolated from smooth muscle, has also been identified in skeletal muscle by IEF/SDS-PAGE of myofibril proteins (25). Antibodies raised against SDS-PAGE-purified desmin from chicken gizzard stain the Z-line region of skeletal muscle myofibrils in indirect immunofluorescence (25, 28). Recently, Granger and Lazarides have shown that desmin exists in a collarlike ring which surrounds each individual Z disk (18). These data suggest that

FIGURE 3 SDS-PAGE of trypsin digested labeled myofibrils (12.5% acrylamide gel). (*a*) Shows the CBB-stained gel. (*b*) Shows the gel as observed with a UV light source. Lanes *1* and *2* are undigested control myofibrils, labeled in the absence (*1*) or presence (*2*) of Ca^{2+}. Myofibrils for this experiment were labeled in the presence of 5 mM GSH. Lanes *3* and *4* show, respectively, the residue and supernate of a 5-min trypsin digestion. Similarly, lanes *5* and *6* correspond to residue and supernate of a 10-min digestion, and lanes *7* and *8* to residue and supernate to a 30-min digestion. Only three large fluorescent fragments (>50,000 daltons) are released into the supernate (lane *b4*), and they possess mol wt of 123,000, 102,000, and 87,000 (arrows adjacent to lane *b3*). The 87,000 fragment is only weakly detectable, but could be discerned in all digests. The 102,000-dalton fragment comigrates with native α-actinin. Comparison of the amounts of 102,000 fragment in the supernate after 5 (lane *b4*), 10 (lane *b6*), or 30 min (lane *b6*) shows a progressive loss of this band, thus suggesting that it is cleaved subsequent to its release from the myofibril. The fluorescent myofibril residues (lanes *b1*, *3*, and *5*) show depletion of most labeled bands.

FIGURE 4 Effect of trypsin on labeled myofibrils. (*a*) One myofibril from a 5-min trypsin digest, showing complete removal of Z-line phase density, with no apparent disruption of other myofibril structures. (*b*) Shows that the Z-line-associated fluorescent labeling has been removed nearly completely. A trace of fluorescence could be detected visually, but was not detectable on film. (*c*) One myofibril from a 10-min trypsin digest chosen to show complete Z-line removal. Arrows denote breaks in the myofibril which occur only at the levels formerly secured by the Z lines. Note that this myofibril also shows some depletion of some A-zone density. (*d*) Shows the complete removal of Z-line fluorescence in this same myofibril. Bar, 5 μm. × 1,600.

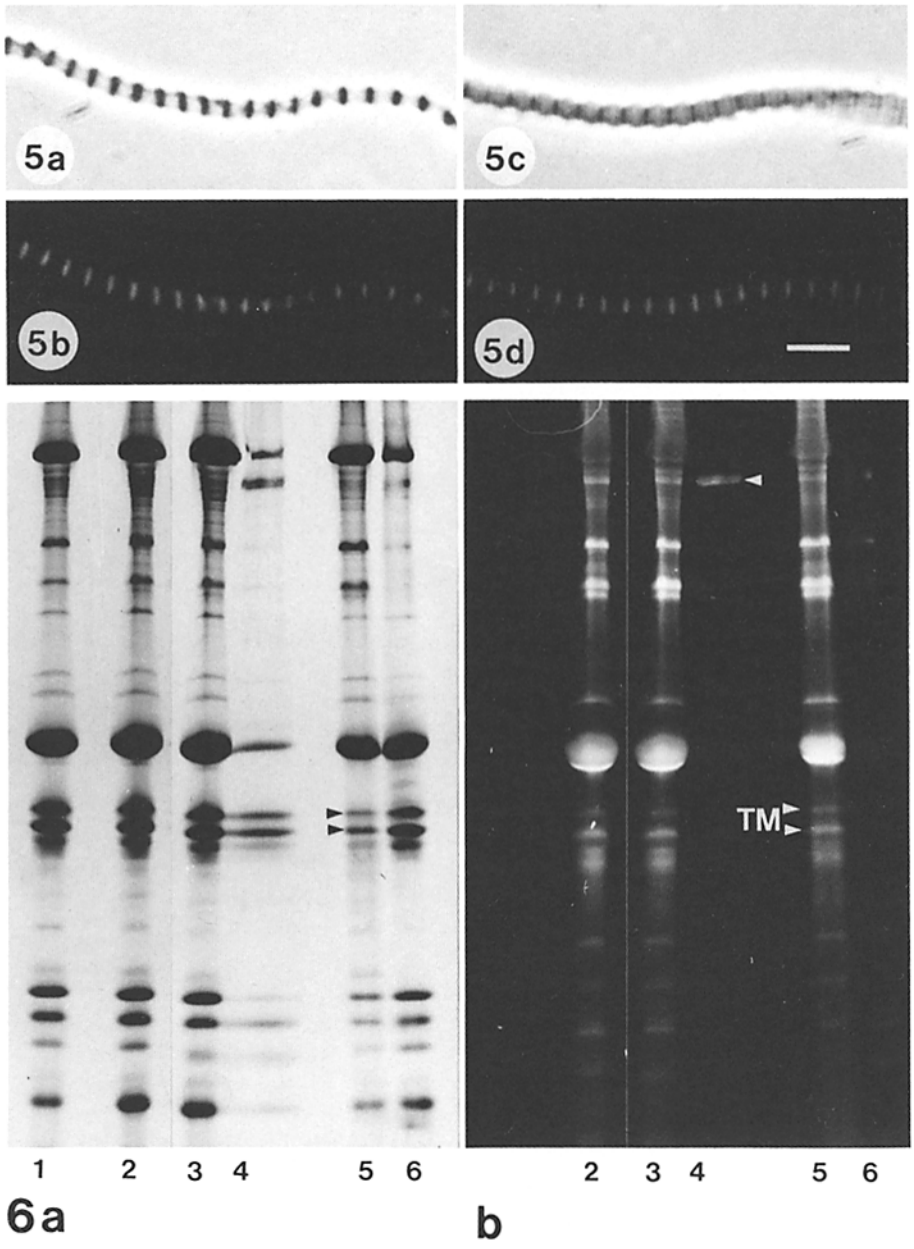


FIGURE 5 High salt extraction of labeled myofibrils. (*a*) Phase microscopy of labeled myofibrils extracted 45 min with 0.6 M KI, showing loss of A- and I-zone density. The sarcomere length appears somewhat shorter than unextracted myofibrils, and the Z-line densities appear somewhat thicker (see text). (*b*) Fluorescence microscopy of the same myofibril shows that the Z-line fluorescence is not extracted by KI. (*c*) Phase microscopy of KCl (plus pyrophosphate)-extracted myofibrils reveals that some of the A-zone density has been removed. Swelling of the myofibril is apparent, giving it a puckered look. (*d*) Fluorescence microscopy shows the retention of the Z-line fluorescence after KCl extraction. Bar, 5 μm. × 1,600.

FIGURE 6 SDS-PAGE analysis of high salt extracts of labeled myofibrils. (*a*) Shows the 17.5% gel stained with CBB. (*b*) Shows the same gel photographed with a UV light source. Lanes *1* and *2* show, respectively, myofibrils labeled in the absence or presence of Ca^{2+}, with 2 mM GSH. Lanes *3* and *4* correspond to the residue and supernate of KCl-extracted, labeled myofibrils. Similarly, lanes *5* and *6* show KI residues and supernate. Note that the 145,000-dalton protein is the only fluorescent species which is extracted in significant amounts by KCl (arrow, *b4*) while CBB staining (*a4*) indicates that some nonfluorescent myosin, actin, and tropomyosins are extracted. Very little fluorescent protein is extracted by KI (*b6*); however CBB staining indicates that large amounts of myosin, actin, and tropomyosin are extracted (*a6*). In particular, note the amounts of nonfluorescent actin and tropomyosin extracted (compare *a6* and *b6*) while fluorescent actin and tropomyosin remain insoluble (compare *a5* and *b5*; tropomyosin is indicated by arrows).

desmin may play an important structural role in the Z line. The labeling of desmin by TGase, in conjunction with the observed Z-line specificity of the labeling reaction, is additional evidence that desmin is localized at the Z line of skeletal muscle myofibrils. Labeled desmin is entirely resistant to extraction with KI, which further implicates it in Z-line structure.

These data provide confirmatory evidence of the Z-line localization of α-actinin and desmin which is not based upon the specificity of an immunological probe. In addition, however, this direct labeling technique provides evidence for the Z-line localization of several other myofibril proteins, including tropomyosin and actin.

There has been considerable controversy over the localization of the tropomyosins in the myofibril Z line. These proteins have not been localized to the Z-line immunologically and do not bind efficiently to myofibrils which have had their Z lines removed by low salt extraction. However, they do appear to enhance the specificity of the α-actinin reconstitution of Z-line density (42, 44). In experiments with the indirect flight muscle from insects, considerable tropomyosin was found in preparations of isolated Z disks, thus suggesting that it may be an integral component of this structure (5, 38). However, none of these experiments adequately proves or disproves the existence of tropomyosin in the Z disk. In our DCAD-labeled myofibrils, the tropomyosins are distinctly identifiable by molecular weight and isoelectric point and constitute one of the major fluorescent protein bands observed. When labeled myofibrils are extracted with 0.6 M KI, a large percentage of the myofibril tropomyosin is solubilized (refer to Fig. 6); little of this solubilized tropomyosin is DCAD labeled, however, and the majority of labeled protein remains insoluble. From this, we conclude that the thin filament tropomyosin is unlabeled and extractable by KI, while the labeled tropomyosin, which is resistant to extraction, is located at or near the myofibril Z line. Any chemical differences between these tropomyosin populations is presently unclear.

The most prominent protein species labeled with DCAD is actin, identified by its mol wt of 42,000 and its isoelectric point. We estimate that nearly one-fifth of the total label incorporated into myofibril protein is present in actin. At this time, we have not identified the specific isoelectric variant of actin which is labeled. However, because α-actin is the predominant actin species found in skeletal muscle (16, 37, 45), we presume that it must represent a portion of the labeled actin. The high level of actin labeling, in conjunction with the Z-line specific fluorescence, leads us to conclude that actin constitutes a major portion of the Z-line structure. Alternatively, a small percentage of thin filament actin immediately adjacent to the Z line may be labeled, accounting for the apparent Z-line labeling observable by fluorescent microscopy.

The former suggestion that actin is a major constituent of the Z line is supported by the high salt extraction data presented above. 0.6 M KI, which readily solubilizes a considerable portion of myofibril actomyosin, leaves the vast majority of labeled actin insoluble (see Fig. 6). Similarly, the fluorescent actin is susceptible to digestion with trypsin under conditions which preferentially remove the myofibril Z line. The hypothesis that actin itself is a major structural protein of the Z disk has not received substantial support in the literature. Part of the difficulty lies in the inability to distinguish Z-line actin from the major portion of actin found in the thin filaments. Immunofluorescence with various antibody preparations directed against actin yields ambiguous results, yet this may be caused by masking of actin antigenicity by α-actinin or other Z-line proteins. Two reports of actin in isolated Z disks of insect flight muscle have been published (5, 38), but it was not determined whether the actin content was because of contamination by thin filament actin during the isolation procedures. A recent report indicates that actin thin filaments underlie the Z-linelike amorphous matrix of nemaline myopathy rod bodies, thus suggesting that these pathological abnormalities may serve as a model for Z-line structure in which actin comprises a major structural protein (47).

There are several proteins which incorporate DCAD to a lesser extent and which are not as easily identifiable. Several of these, most notably two sets of high molecular weight proteins, a 145,000 protein doublet, and several low molecular weight proteins, are consistently present in our labeled myofibril preparations. These proteins are resistant to extraction by extensive washing with PBS and are not extracted by 5% NP-40 (unpublished observations). Contamination by sarcoplasmic reticulum (SR) and T-system membrane proteins is an inherent difficulty in working with isolated myofibrils from mammalian sources. By using fluorescent probes which bind to hydro-

phobic environments, we have shown that even well washed myofibrils contain hydrophobic regions, presumably membrane remnants of the SR and T system. Extraction with 0.5% NP-40 was sufficient to abolish binding of the probes, thus suggesting the removal of the membrane fragments (27). In light of this result, it seems unlikely that these DCAD-labeled proteins are actually membrane protein contaminants from the SR or T system. However, this possibility, or the possibility of tenacious adsorption of sarcoplasmic components, cannot be eliminated entirely.

The 145,000-mol wt protein labeled by TGase may be identical to the C protein described by Etlinger et al. (12). This conclusion is based upon the similarities in molecular weight, and the fact that some labeled myofibrils show a weak fluorescence pattern consistent with the A-zone localization of the C protein. In addition, the labeling intensity of this protein increases as the glutathione concentration of the labeling media is increased, a manipulation which also results in solid labeling of the A zone, with appearance of labeled myosin heavy chains, and light chains (data not shown).

A substantial amount of myofibrillar material solubilized by SDS and reducing agents fails to enter the 4% acrylamide stacking gel employed, thus suggesting that this material possesses an extremely high molecular weight. Similar high molecular weight components have been previously reported in preparations of vertebrate myofibrils and in isolated Z disks from insect muscle (5, 11, 12, 38). Fig. 2 shows that a substantial amount of DCAD is incorporated into this high molecular weight material, and into additional material which penetrates the stacking gel, but not the resolving gels employed. The intensity of the fluorescence, when related to microscopy of the myofibrils, suggests that this material originates from the Z line. Its extreme molecular weight in the presence of denaturing and reducing agents suggests that it may consist of Z-line proteins covalently cross linked through a bond other than a disulfide, perhaps reflecting the action of an endogenous muscle TGase.

In addition to the above proteins which are invariant in their labeling characteristics, there are several proteins whose presence or labeling characteristics vary. These proteins possess mol wt of 87,000 and 68,000 and have not been identified as any known myofibril constituents. A protein with a mol wt of 87,000 has been found in isolated Z disks from two insect sources (5, 38), and an 85,000 protein is found in DOC extractions of rabbit myofibrils (11), suggesting that there may be a Z-line component with this molecular weight. This variability observed in our myofibril preparations suggests that these proteins may be degradation products of other myofibril or Z-line proteins.

Guinea pig liver TGase adsorbs tenaciously to isolated chick myofibrils, and is not dissociated by extensive washing with PBS, 0.6 M KI, or 5% NP-40. This adsorption is independent of Ca^{2+}, which is required for enzyme activity. We have not determined whether this binding is caused by a nonspecific affinity of the enzyme for the myofibril Z line. However, when activated by Ca^{2+}, bound TGase incorporates DCAD label. Because fluorescence is Z-line specific, active TGase must be localized strictly at the Z line.

We thank Mr. David R. Balzer, Jr., for his assistance with two-dimensional gel electrophoresis.

This work was supported by grant PHS GM 06965-18 from the National Institutes of Health, and also by grants from the Muscular Dystrophy Association of America, and the American Cancer Society. David L. Gard was also supported by a National Science Foundation Predoctoral Fellowship.

Received for publication 17 April 1978, and in revised form 18 December 1978.

REFERENCES

1. ABERNATHY, J. L., R. L. HILL, and L. P. GOLDSMITH. 1977. ε-(γ-glutamyl) lysine cross-links in human stratum corneum. *J. Biol. Chem.* **252**:1837–1839.
2. ANDERSON, D. R., J. L. DAVIS, and K. L. CARRAWAY. 1977. Calcium-promoted change of the human erythrocyte membrane. *J. Biol. Chem.* **252**:6617–6623.
3. BIRCKBICHLER, P. J., R. M. DOWBEN, S. MATACIC, and A. G. LOEWY. 1973. Isopeptide bonds in membrane proteins from eukaryotic cells. *Biochim. Biophys. Acta.* **291**:149–155.
4. BIRCKBICHLER, P. J., G. R. ORR, and H. A. CARTER. 1977. Catalytic formation of ε-(γ-glutamyl) lysine in guinea pig liver transglutaminase. *Biochem. Biophys. Res. Commun.* **78**:1–7.
5. BULLARD, B., and G. N. SAINSBURY. 1977. The proteins of the Z line of insect flight muscle. *Biochem. J.* **161**:399–403.
6. CHUNG, S. I. 1972. Comparative studies on tissue transglutaminase and factor XIII. *Ann. N. Y. Acad. Sci.* **202**:240–255.
7. CHUNG, S. I., and J. E. FOLK. 1972. Transglutaminase from hair follicle of guinea pig. *Proc. Natl. Acad. Sci. U.S.A.* **69**:303–307.
8. CONNELLAN, J. M., S. I. CHUNG, N. K. WHETZEL, L. M. BRADLEY, and J. E. FOLK. 1971. Structural properties of guinea pig liver transglutaminase. *J. Biol. Chem.* **246**:1093–1098.
9. DUTTON, A., and S. I. SINGER. 1975. Cross-linking and labeling of membrane proteins by transglutaminase-catalyzed reactions. *Proc. Natl. Acad. Sci. U. S. A.* **72**:2568–2571.
10. EBASHI, S., and F. EBASHI. 1965. α-Actinin, a new structural protein from striated muscle. I. Preparation and action on actomyosin-ATP interaction. *J. Biochem. (Tokyo).* **58**:7–12.
11. ETLINGER, J. D., and D. A. FISCHMAN. 1973. M and Z band components and the assembly of myofibrils. *Cold Spring Harbor Symp. Quant. Biol.* **37**:511–522.
12. ETLINGER, J. D., R. ZAK, and D. A. FISCHMAN. 1976. Compositional

studies of myofibrils from rabbit striated muscle. *J. Cell Biol.* **68:**123–141.
13. EVANS, R. M., and L. M. FINK. 1977. Identification of transmembrane bridging proteins in the plasma membrane of cultured mouse L-cells. *Proc. Natl. Acad. Sci. U. S. A.* **74:**5341–5344.
14. FOLK, J. E., and S. I. CHUNG. 1973. Molecular and catalytic properties of transglutaminases. *Adv. Enzymol.* **38:**109–191.
15. FOLK, J. E., and P. W. COLE. 1966. Mechanism of action of guinea pig liver transglutaminase. *J. Biol. Chem.* **241:**5518–5525.
16. GARRELS, J. I., and W. GIBSON. 1976. Identification and characterization of multiple forms of actin. *Cell.* **9:**793–805.
17. GOLL, D. E., W. F. H. M. MOMMAERTS, M. K. REEDY, and K. SERAYDARIAN. 1969. Studies on α-actinin-like proteins liberated during trypsin digestion of α-actinin and of myofibrils. *Biochim. Biophys. Acta.* **175:**174–194.
18. GRANGER, B. L., and E. LAZARIDES. 1978. The existence of an insoluble Z disc scaffold in chicken skeletal muscle. *Cell.* **15:**1253–1268.
19. HOLTZER, H. 1959. Some further uses of antibodies for analyzing the structure and development of muscle. *Exp. Cell. Res.* **7**(Suppl.):234.
20. HUBBARD, B. D., and E. LAZARIDES. 1979. Copurification of actin and desmin from chicken smooth muscle and their copolymerization in vitro to intermediate filaments. *J. Cell Biol.* **80:**166–182.
21. HUXLEY, H. E. 1963. Electron microscopic studies on the structure of natural and synthetic protein filaments from striated muscle. *J. Mol. Biol.* **7:**281–308.
22. IWANJ, V. 1977. The use of liver transglutaminase for protein labeling. *Eur. J. Biochem.* **80:**359–368.
23. KELLY, D. E., and M. A. CAHILL. 1972. Filamentous and matrix components of skeletal muscle Z disks. *Anat. Rec.* **174:**623–642.
24. LANE, B. P., J. ELIAS, and E. DRUMMOND. 1977. Immunoelectron microscopic localization of α-actinin in skeletal muscle cells. *J. Histochem. Cytochem.* **25:**69–72.
25. LAZARIDES, E., and D. R. BALZER, JR. 1978. Specificity of desmin to avian and mammalian muscle cells. *Cell.* **14:**429–438.
26. LAZARIDES, E., and K. BURRIDGE. 1975. α-Actinin: immunofluorescent localization of a muscle structural protein in nonmuscle cells. *Cell.* **6:**289–298.
27. LAZARIDES, E., and B. L. GRANGER. 1978. Fluorescent localization of membrane sites in glycerinated chicken skeletal muscle fibers and the relationship of these sites to the protein composition of the Z disc. *Proc. Natl. Acad. Sci. U. S. A.* **75:**3683–3687.
28. LAZARIDES, E., and B. D. HUBBARD. 1976. Immunological characterization of the 100 Å filaments from muscle cells. *Proc. Natl. Acad. Sci. U. S. A.* **73:**4344–4348.
29. LORAND, L., and K. KONISHI. 1964. Activation of the fibrin stabilizing factor of plasma by thrombin. *Arch. Biochem. Biophys.* **105:**58–67.
30. LORAND, L., R. SHISHIDO, K. N. PARAMESWARAN, and T. L. STECK. 1975. Modification of human erythrocyte ghosts with transglutaminase. *Biochem. Biophys. Res. Commun.* **67:**1158–1166.
31. LORAND, L., L. B. WEISSMAN, D. L. EPEL, and J. BRUNER-LORAND. 1976. Role of the intrinsic transglutaminase in the Ca^{2+}-mediated cross-linking of erythrocyte proteins. *Proc. Natl. Acad. Sci. U. S. A.* **73:**4479–4481.
32. MASAKI, T., M. ENDO, and S. EBASHI. 1967. Localization of 6S component of α-actinin at Z band. *J. Biochem. (Tokyo).* **62:**630–632.
33. O'FARRELL, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250:**4007–4021.
34. OKUMURA, T., and G. A. JAMIESON. 1976. Platelet glycocalicin: Orientation of glycoproteins of the human platelet surface. *J. Biol. Chem.* **251:**5944–5949.
35. RICE, R. H., and H. GREEN. 1977. The cornified envelope of terminally differentiated human epidermal keratinocytes consists of cross-linked protein. *Cell.* **11:**417–422.
36. RICE, R. H., and H. GREEN. 1978. Relation of protein synthesis and transglutaminase activity to formation of the cross-linked envelope during terminal differentiation of the cultured human epidermal keratinocyte. *J. Cell Biol.* **76:**705–711.
37. RUBENSTEIN, P. A., and J. A. SPUDICH. 1977. Actin microheterogeneity in chick embryo fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* **74:**120–123.
38. SAIDE, J. D., and W. C. ULLRICK. 1974. Purification and properties of the isolated honey bee Z disk. *J. Mol. Biol.* **87:**671–683.
39. SAUKER, N. K., D. D. CLARKE, and H. WAELSCH. 1957. An enzymically catalyzed incorporation of amines into proteins. *Biochim. Biophys. Acta.* **251:**451–452.
40. SCHACTERLE, G. R., and R. L. POLLACK. 1973. A simplified method for the quantitative assay of small amounts of protein in biological material. *Anal. Biochem.* **51:**654–655.
41. SCHWARTZ, M. L., S. V. PIZZO, R. L. HILL, and P. A. MCKEE. 1973. Human factor XIII from plasma and platelets: Molecular weights, subunit structures, proteolytic activation, and cross-linking of fibrinogen and fibrin. *J. Biol. Chem.* **248:**1395–1407.
42. STROMER, M. H., and D. E. GOLL. 1972. Studies on purified α-actinin II. Electron microscopic studies on the competitive binding of α-actinin and tropomyosin to Z line extracted myofibrils. *J. Mol. Biol.* **67:**489–494.
43. STROMER, M. H., D. E. GOLL, and L. E. ROTH. 1967. Morphology of rigor-shortened bovine muscle and the effect of trypsin on pre- and postrigor myofibrils. *J. Cell Biol.* **34:**431–445.
44. STROMER, M. H., D. J. HARTSHORNE, H. MUELLER, and R. V. RICE. 1969. Effect of various protein fractions on Z- and M-line reconstitution. *J. Cell Biol.* **40:**167–178.
45. WHALEN, R. G., G. S. BUTLER-BROWNE, and F. GROSS. 1976. Protein synthesis and actin heterogeneity in calf muscle cells in culture. *Proc. Natl. Acad. Sci. U. S. A.* **73:**2018–2022.
46. WILLIAMS-ASHMAN, H. G., A. C. NOTIDES, S. S. PABALAN, and L. LORAND. 1972. Transglutaminase reactions involved in the enzymatic coagulation of semen: Isolation of ε-(γ-glutamyl) lysine dipeptide from clotted secretion protein of guinea pig seminal vesicle. *Proc. Natl. Acad. Sci. U. S. A.* **69:**2322–2325.
47. YAMAGUCHI, M., R. M. ROBSON, M. H. STROMER, D. S. DAHL, and T. ODA. 1978. Actin filaments form the backbone of nemaline myopathy rods. *Nature (Lond.).* **271:**265–267.